



# Prevention of Vascular Graft Infections with Antibiotic Graft Impregnation Prior to Implantation: *In Vitro* Comparison between Daptomycin, Rifampin and Nebacetin<sup>☆</sup>

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## WHAT THIS PAPER ADDS

- Antibiotic impregnation of vascular grafts prior to implantation for local antibiotic release remains an adjuvant measure against bacterial colonisation. However, controversial results between different antibiotic agents have been established. Major drawbacks remain the limited antibacterial effect against high-virulent Gram-negative micro-organisms, the selective antibacterial activity (e.g., against only Gram-positive micro-organisms) and the rapid development of bacterial resistance against antibiotics.
- This study introduces nebacetin as an effective and safe antibiotic agent for vascular graft impregnation against both Gram-positive and -negative micro-organisms. In this context, impregnation with nebacetin will contribute to reduction of perioperative vascular graft infections and optimisation of their challenging treatment.

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## ABSTRACT

**Objective:** To compare the *in vitro* efficacy of graft impregnation with nebacetin versus rifampin versus daptomycin against vascular graft infections caused by *Staphylococcus epidermidis* and *Staphylococcus aureus* and nebacetin versus rifampin against *Pseudomonas aeruginosa* and *Escherichia coli*.

**Materials:** Twenty-three Dacron-grafts (1 cm<sup>2</sup>) for each micro-organism were microbiologically tested and eight grafts per antibiotic underwent viability tests against human umbilical vein endothelial cells (ECs). Fifteen grafts (5/antibiotic agent) underwent 15 min impregnation and contamination with 4 ml bacterial solution (optical density (OD<sub>600 nm</sub>): 0.20 ± 0.02). After 24-h-incubation, all grafts were washed with phosphate-buffered saline and underwent sonification to release viable adherent bacteria. OD<sub>600 nm</sub> of the solution was measured. Afterwards, six 1:10 dilution steps took place and colony-forming units (CFUs) were counted.

**Results:** Nebacetin showed comparable efficacy to daptomycin against Gram-positive bacteria. Both eradicated more efficiently *S. epidermidis* than rifampin (daptomycin:0, rifampin:5 ± 7.3, nebacetin:0 CFU ml<sup>-1</sup>, *P* = 0.0003). All antibiotics showed comparable antibacterial activity against *S. aureus*. Nebacetin was more efficient than rifampin to eradicate Gram-negative organisms (*P. aeruginosa*: rifampin:1308 ± 252, nebacetin:8 ± 8 CFU ml<sup>-1</sup>, *P* = 0.01, *E. coli*: rifampin:294 ± 159, nebacetin:0.2 ± 0.5 CFU ml<sup>-1</sup>, *P* = 0.001), while only rifampin was toxic against ECs (daptomycin:30.88 ± 5.44, rifampin:5.13 ± 5.08, nebacetin:28.50 ± 3.82 ECs/field, *P* = 0.0003).

**Conclusions:** Nebacetin showed excellent *in vitro* antibacterial activity against both Gram-positive and -negative pathogens representing an effective candidate for vascular graft impregnation.

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Infection of surgical implants represents a devastating complication with essential clinical and economical impact.<sup>1</sup> In vascular surgery, the average rate of graft infections is about 4% with high morbidity and mortality rates (10–75%) and an estimated average cost of combined medical and surgical treatment amounted to €30 000.<sup>1–4</sup> Besides, the changing epidemiology of infections and

the unattended use of antibiotics led to an increased incidence of antibiotic resistance of otherwise harmless micro-organisms such as *Staphylococcus aureus* and *Staphylococcus epidermidis*.<sup>2,3</sup> Additionally, vascular graft infections (VGIs) caused by Gram-negative organisms such as *Pseudomonas aeruginosa* are associated with high reinfection rates and poor survival outcomes.<sup>4,5</sup>

Notwithstanding on late VGI, the majority of early VGI is caused by inoculation of bacteria at the time of surgery despite perioperative antibiotic prophylaxis.<sup>2</sup> It is well known that, in this case, intravenous antibiotic therapy is unable to reach a sufficient topical tissue concentration to penetrate the biofilm which protects the embedded bacteria.<sup>6</sup> Therefore, impregnation of vascular prostheses with antibiotic agents prior to implantation for local antibiotic release seems advantageous.<sup>6–8</sup> However, controversial results about the most effective antibiotic agent have been published. Gold standard for graft impregnation or coating remains rifampin.<sup>8</sup> Currently, daptomycin showed excellent antibacterial effect on biofilm-associated cells, especially when combined with rifampin.<sup>8</sup> However, our clinical experience with another antibiotic agent, which combines neomycin and bacitracin (i.e., nebacetin), for the treatment of VGI revealed encouraging results as well.<sup>9,10</sup>

We hypothesised that nebacetin is more efficient than daptomycin or rifampin for the *in vitro* prevention of VGI. Thus, we compared all three antibiotics in an experimental model of graft infection caused by *S. epidermidis*, *S. aureus* (as Gram-positive pathogens) and nebacetin versus rifampin for graft infection caused by *P. aeruginosa* and *Escherichia coli* (as Gram-negative pathogens).

## Materials and Methods

### Bacterial strain

The bacterial isolates in this study were 20044DSMZ for *S. epidermidis*, 20231DSMZ for *S. aureus*, ATCC27853 for *P. aeruginosa* and 1103DSMZ for *E. coli*. Strains were sub-cultured on tryptic soy agar according to the recommendations of the German Collection of Microorganisms and Cell Cultures GmbH ([www.dsmz.de](http://www.dsmz.de)). The optical density (OD<sub>600 nm</sub>), used for graft contamination, amounted to 0.20. This OD corresponds to  $1 \times 10^6$  colony-forming units (CFUs) per millilitre for *S. epidermidis*,  $1 \times 10^8$  CFU ml<sup>-1</sup> for *S. aureus*,  $1 \times 10^7$  CFU ml<sup>-1</sup> for *P. aeruginosa* and  $1 \times 10^7$  CFU for *E. coli*.

### Antibiotic agents

Sterile solution preparation of daptomycin 500 mg (Cubicin®, Cubist Pharmaceuticals, Inc, Lexington, USA), rifampin 600 mg (Eremfat®, Riemser Arzneimittel, Greifswald, Germany) and nebacetin 100 ml (bacitracin 250 IU, neomycin sulphate 5000 IU, Baneocin pro instillatione®, Sandoz, Kundl, Switzerland) was performed according to manufacturer's instructions. The antibiotic concentrations were the same with the respective concentrations achievable in humans.

### Grafts

Commercially available double-woven velour vascular grafts (Unigraft®, BBraun, Melsungen, Germany) made of fine polyester fibres and coated with absorbable modified gelatine were used. The grafts were processed to  $1 \times 1$  cm pieces under aseptic conditions.

### Experimental protocol

The grafts were processed to a total number of 129 pieces ( $n = 129$ ). Twenty-three 1-cm<sup>2</sup> grafts were used for the

microbiological investigations against *S. aureus* and *S. epidermidis*, respectively, 18 grafts (daptomycin was not tested for Gram-negative micro-organisms) against *P. aeruginosa* and *E. coli*, respectively ( $n = 82$  grafts) and eight grafts per antibiotic agent ( $n = 32$  grafts) for viability tests against human umbilical cord vein-derived endothelial cells (ECs). Additionally, we used five grafts per antibiotic agent ( $n = 15$ ) to assess the amount (A) of the antibiotic that has been impregnated into each graft ( $A = W_{AI} - W_{BI}$ ,  $W_{AI}$ : weight of graft after impregnation and  $W_{BI}$ : weight before impregnation).

An overview of our experiment protocol is illustrated in Fig. 1. In detail, the impregnation of the grafts (phase I) was done in 50-ml Falcon tubes containing the antibiotic solution and lasted 15 min for each specimen. The impregnation time was adopted from clinical practice. Then, 4 ml of tryptic soy broth (TSB, Oxoid, UK) were added under aseptic conditions to a Petri dish and contaminated with bacteria (OD<sub>600 nm</sub>:  $0.20 \pm 0.02$ ). Each impregnated graft was placed in a separate Petri dish.

In the second phase, five grafts ( $n = 5$ ) per antibiotic agent were placed into the incubator (37 °C, 5%CO<sub>2</sub>) for 24 h. Two untreated grafts for each micro-organism ( $n = 2$ , positive control), one untreated graft without contamination ( $n = 1$ , negative control) and a Petri dish only with TSB (to assess our medium sterility) were used as controls. Three grafts ( $n = 3$ ) for each antibiotic were also placed into the incubator for 24 h and used for the assessment of the respective biofilms through scanning electron microscopy (SEM). SEM evaluation underwent additionally one untreated graft with micro-organism ( $n = 1$ , positive control) and one graft without micro-organism or antibiotic ( $n = 1$ , negative control). After incubation, all grafts were removed and washed three times in 20-ml phosphate-buffered saline (PBS) from GIBCO® (Darmstadt, Germany) to eliminate unbound bacteria. Thereafter, viable adherent bacteria were released by sonification on low power (100%) for 20 min. OD<sub>600 nm</sub> of PBS containing the dislodged bacteria was measured over again. We performed six 1:10 dilution steps of the solution and 100 µL of each dilution were plated onto TSB agar plates. All plates were incubated overnight at 37 °C and colonies were visually identified and counted.

The third phase included viability tests for ECs. Regional ethics committee approved the cell isolation from healthy new-born donors as previously described<sup>11</sup> and their culture at 5%CO<sub>2</sub> and 37 °C and in supplemented endothelial growth medium No.2 (Lonza, Basel, Switzerland) in gelatin-coated (1%) flasks (Nunc, Langenselbold, Germany). Upon confluence, cells were passaged with trypsin/ethylenediaminetetraacetic acid and the total cell number was determined by using a Casy electric field multi-channel cell counter (Roche Innovatis Reutlingen, Germany). Subsequently, the desired number of cells was seeded into gelatine-coated 24-well culture plates for the experiment.

On the day of experiment, the cell culture medium was renewed and the antibiotic solutions were freshly prepared. Twenty-four grafts ( $n = 24$ ) were impregnated with antibiotic (eight grafts per antibiotic) and eight untreated grafts were placed in PBS serving as control group. After 15 min of antibiotic impregnation, graft pieces were transferred into the wells of the cell culture dishes and incubated for 24 h (37 °C, 5%CO<sub>2</sub>) until the cell viability analysis was performed. The number of viable ECs was assessed by fluorescent-live cell imaging using calcein acetoxymethyl ester – a green fluorescent dye that stains living cells via cleavage by non-specific intracellular esterases. The graft pieces as well as the medium were discarded and each well was washed 3 times with PBS. Then, ECs were stained with calcein acetoxymethyl ester (Invitrogen) at a final concentration of 2 µM for 30 min at 37 °C. Next, the staining solution was replaced with calcium-containing PBS and photomicrographs were taken using an AxioCam-MRm camera (Zeiss, Jena,

Germany) connected to an AxioObserver A1-microscope using AxioVision software. For each well, photomicrographs of five random low power fields were taken and the number of adherent viable cells was determined by counting the positively stained ECs per microscopic field.

#### Scanning electron microscopy

The grafts for SEM were also washed three times in PBS in order to remove planktonic cell cultures (suspended micro-organisms) from Petri dish, which might cause diagnostic bias in biofilm observation. All grafts were fixed in 2.5% glutaraldehyde and sodium cacodylate buffer (0.2 M, pH 7.3) and transported in 50-ml Falcon tubes into a sterile container to laser centre, and 24 h later were observed in the SEM (FEI Quanta 400). This microscope increases its imaging capabilities with high-resolution high-, low- and extended low-vacuum (ESEM) secondary electron imaging. In our experiment, a voltage of 30 kV and a resolution of 1 nm were used. Our goal was a descriptive assessment of biofilms of each micro-organism and the antibiotic effect on biofilm formation supportively to our microbiological results. 'Microbial biofilm' was defined as populations of micro-organisms that are concentrated at an interface (in this case solid) and typically surrounded by an extracellular polymeric substance.<sup>12</sup>

#### Statistical analysis

All analyses were performed and graphs were created with GraphPad 5.0d Software, Inc. Quantitative culture results were

presented as arithmetic mean  $\pm$  standard deviation (SD). This study could not be blinded due to the orange colour of rifampin. Sample sizes were estimated through Mead's resource equation.<sup>13</sup> Comparisons between two groups were performed using Mann–Whitney test. We did not perform any normality test to assess Gaussian distributions due to the small samples being compared. Thus, comparison between three groups (daptomycin vs. rifampin vs. nebacetin) was done using Kruskal–Wallis' analysis of variance.<sup>14</sup> Differences at  $P < 0.05$  were considered statistically significant.

## Results

### Amount of impregnated antibiotic

According to the aforementioned formula ( $A = W_{AI} - W_{BI}$ ), the mean amount of the impregnated antibiotic within the grafts was  $30 \pm 5$  mg for daptomycin,  $35 \pm 4$  mg for rifampin and  $28 \pm 8$  mg for nebacetin. Comparably, in the commercially available susceptibility tests for ordinary drug resistance the loaded amount of these antibiotics is  $0.03 \text{ mg ml}^{-1}$  for daptomycin,  $0.01 \text{ mg ml}^{-1}$  for neomycin and bacitracin, respectively, and  $0.005 \text{ mg ml}^{-1}$  for rifampin.

### Staphylococcus epidermidis

OD<sub>600 nm</sub> after 24-h incubation and prior to sonification: Mean OD<sub>600</sub> was  $0.04 (\pm 0.01)$  in the daptomycin group (DG),  $0.18 \pm 0.02$

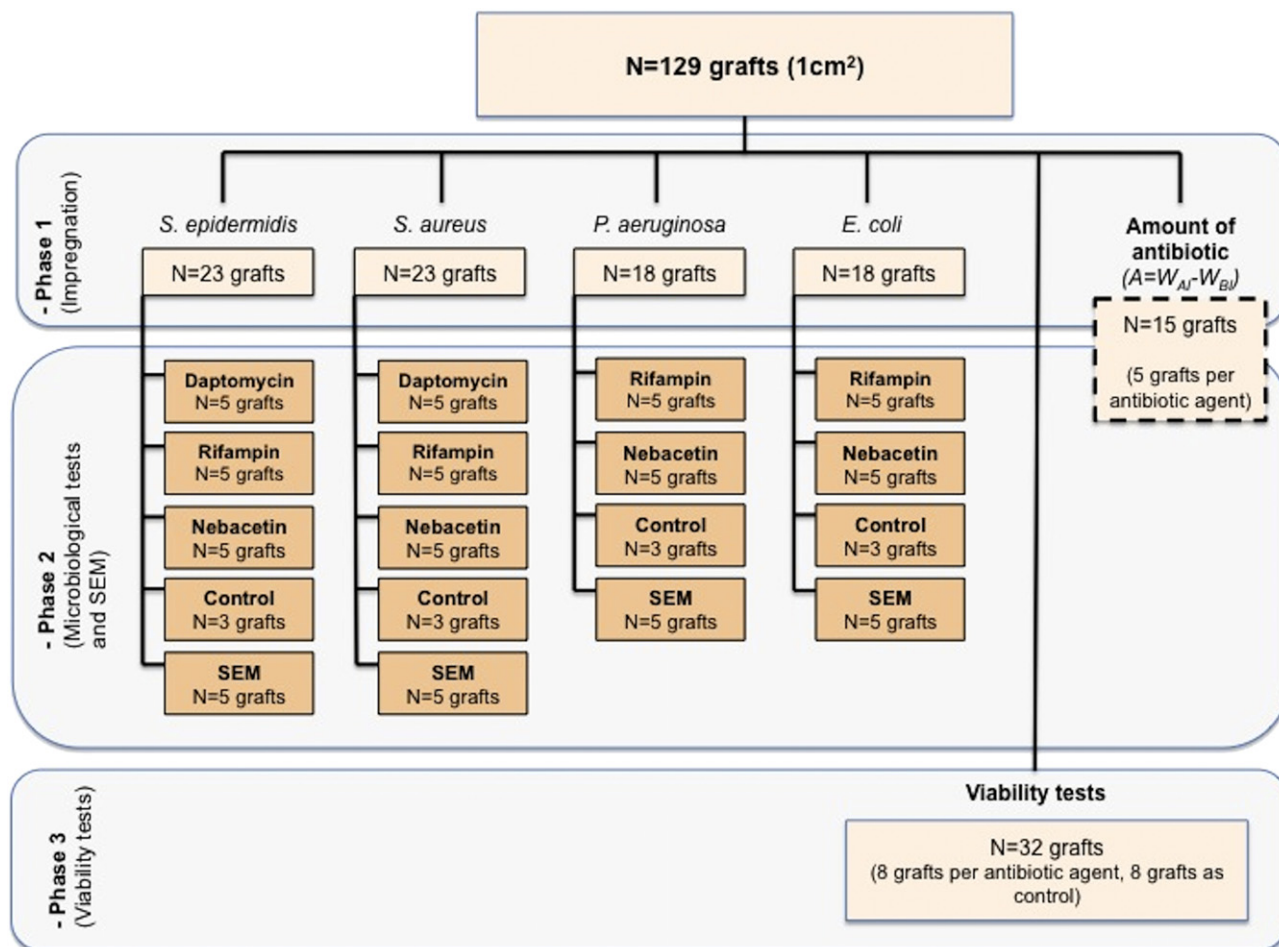


Figure 1. Schematic overview of our experimental study protocol.

in the rifampin group (RG) and  $0.03 \pm 0.002$  in the nebacetin group (NG) [ $P < 0.007$ , Kruskal Wallis statistic (KWS): 9.9].

**OD<sub>600 nm</sub> and bacterial count after sonification:** Mean OD after sonification is presented in Fig. 2A. The average bacterial count was measured after second and sixth dilution. Regarding second dilution, bacterial count was 0 CFU ml<sup>-1</sup> in the DG and the NG and  $5 \pm 7.3$  CFU ml<sup>-1</sup> in the RG ( $P = 0.0003$ , KWS: 16.4). The positive control grafts showed  $3332 \pm 662$  CFU ml<sup>-1</sup>. After sixth dilution, average bacteria count was 0 CFU ml<sup>-1</sup> in the DG and the NG and  $0.2 \pm 0.4$  CFU ml<sup>-1</sup> in the RG ( $P = 0.12$ , KWS:4.2). The positive control grafts showed  $64 \pm 30$  CFU ml<sup>-1</sup> after sixth dilution. Negative controls as well as medium remained negative for microorganisms after all dilution steps.

#### *Staphylococcus aureus*

**OD<sub>600 nm</sub> after 24-h incubation and prior to sonification:** After 24-h incubation with *S. aureus*, mean OD<sub>600</sub> was  $0.4 \pm 0.1$  in the DG,  $0.6 \pm 0.1$  in the RG and  $0.3 \pm 0.4$  in the NG ( $P = 0.06$ , KWS:5.7).

**OD<sub>600 nm</sub> and bacterial count after sonification:** After sonification, mean OD<sub>600</sub> is presented in Fig. 2B. The average bacterial count after second as well as sixth dilution was 0 CFU ml<sup>-1</sup> in all antibiotic groups. By the positive control, the bacteria count was not measurable after second dilution step due to massive bacterial 'slime'. However, after sixth dilution average bacteria count in the positive control amounted to  $49 \pm 43$  CFU ml<sup>-1</sup>. Negative controls as well as medium remained negative.

#### *Pseudomonas aeruginosa*

**OD<sub>600 nm</sub> after 24-h incubation and prior to sonification:** TSB after 24-h incubation and before sonification revealed a mean OD<sub>600</sub> of  $0.4 \pm 0.4$  in the RG and  $0.17 \pm 0.03$  in the NG ( $P = 0.06$ ).

**OD<sub>600 nm</sub> and bacterial count after sonification:** Mean OD<sub>600</sub> after sonification is illustrated in Fig. 2C. The average count of

adherent bacteria after the second dilution was  $1308 \pm 252$  in the RG and  $8 \pm 8$  in the NG ( $P = 0.01$ ). After sixth dilution, the RG showed  $241 \pm 127$  CFU ml<sup>-1</sup> and the NG 0 CFU ml<sup>-1</sup> ( $P = 0.008$ ). According to the positive control, bacteria count was not measurable after second dilution step; however, after sixth dilution the average count amounted to  $149 \pm 200$  CFU ml<sup>-1</sup>. Negative controls as well as medium remained negative.

#### *Escherichia coli*

**OD<sub>600 nm</sub> after 24-h incubation and prior to sonification:** Mean OD<sub>600</sub> of the TSB solution was  $1.7 \pm 0.2$  in the RG and  $0.4 \pm 0.1$  in the NG ( $P = 0.008$ ).

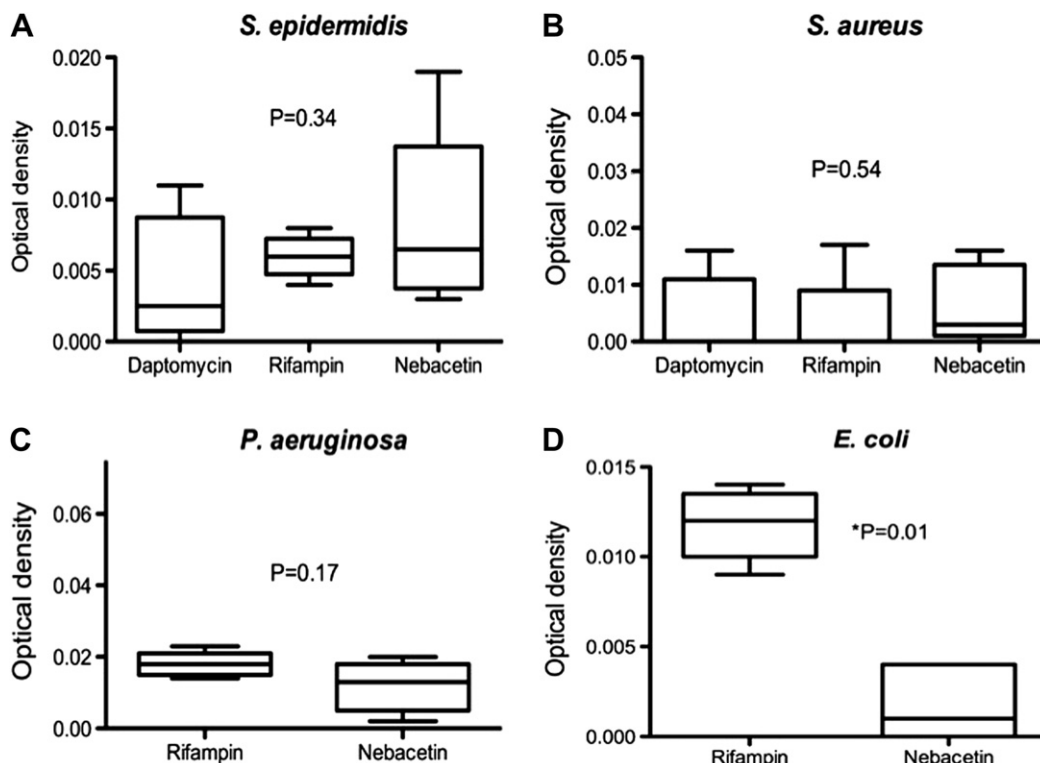
**OD<sub>600 nm</sub> and bacterial count after sonification:** Mean OD<sub>600</sub> after sonification is presented in Fig. 2D. The average count of adherent bacteria was measured after second and sixth dilution. After second dilution, the average bacteria count was  $294 \pm 159$  CFU ml<sup>-1</sup> in the RG and  $0.2 \pm 0.5$  CFU ml<sup>-1</sup> in the NG ( $P = 0.001$ ). The CFUs in the positive control grafts were not measurable. After sixth dilution, average bacteria count amounted to  $2.4 \pm 1.2$  in the RG and 0 CFU ml<sup>-1</sup> in the NG ( $P = 0.03$ ). The positive control grafts showed  $232 \pm 318$  CFU ml<sup>-1</sup> after sixth dilution. Negative controls and medium remained negative during all dilution steps.

#### Viability tests for ECs

The optical as well as the arithmetic results of the viability tests are illustrated in Fig. 3

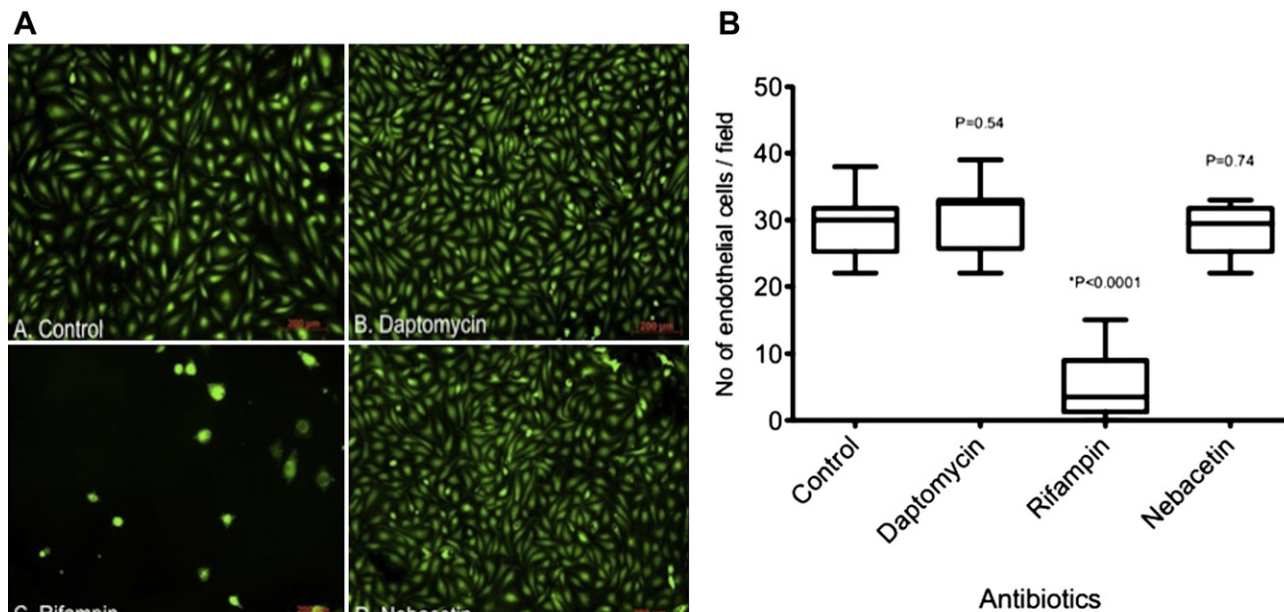
#### Biofilm formation

Bacterial biofilms were visible in all positive controls in SEM. In Figs. 4–7, we demonstrate the antibacterial effect of the respective antibiotics compared to positive controls. Concerning Gram-

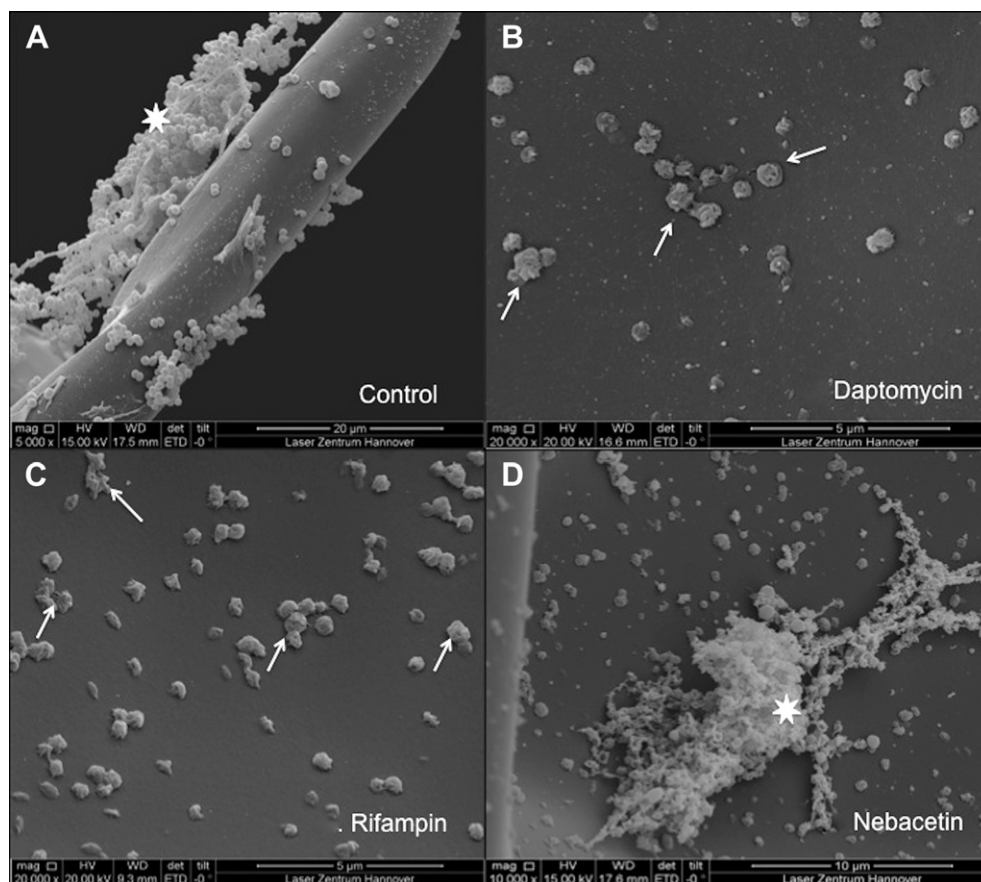


**Figure 2.** Optical density (at 600 nm) of TSB after 24h incubation and after sonification of the grafts for (A) *S. epidermidis*, (B) *S. aureus*, (C) *P. aeruginosa* and (D) *E. coli*.





**Figure 3.** (A) Viable endothelial cells stained with calcein acetoxytmethyl ester under optical microscope ( $\times 10$  magnification) after addition of antibiotic-impregnated grafts and 24h incubation at 37 °C and 5% CO<sub>2</sub> and (B) Average count of viable endothelial cells after addition of antibiotic-impregnated grafts and 24h incubation (37 °C, 5%CO<sub>2</sub>) reflecting the toxicity of each antibiotic agent.



**Figure 4.** Example of a 24h-old biofilm of *S. epidermidis* in control group (A), after impregnation with daptomycin (B), rifampin (C) and nebaceticin (D). The asterisks show the formed biofilm with intact organisms and the arrows represent the eliminated bacteria (lysis).

positive micro-organisms, all antibiotics led to an extensive bacterial lysis. However, we observed a few number of intact biofilms within the fibres of grafts that were impregnated with rifampin and nebacetin (Figs. 4 and 5), whereas daptomycin-impregnated grafts were free from any biofilm. Regarding Gram-negative micro-organisms, only nebacetin showed successful elimination of both tested micro-organisms (Figs. 6 and 7). Noteworthy is that nebacetin led to elimination of *E. coli* even within intact biofilms of the micro-organism and even if its characteristic network had been built (Fig. 7D).

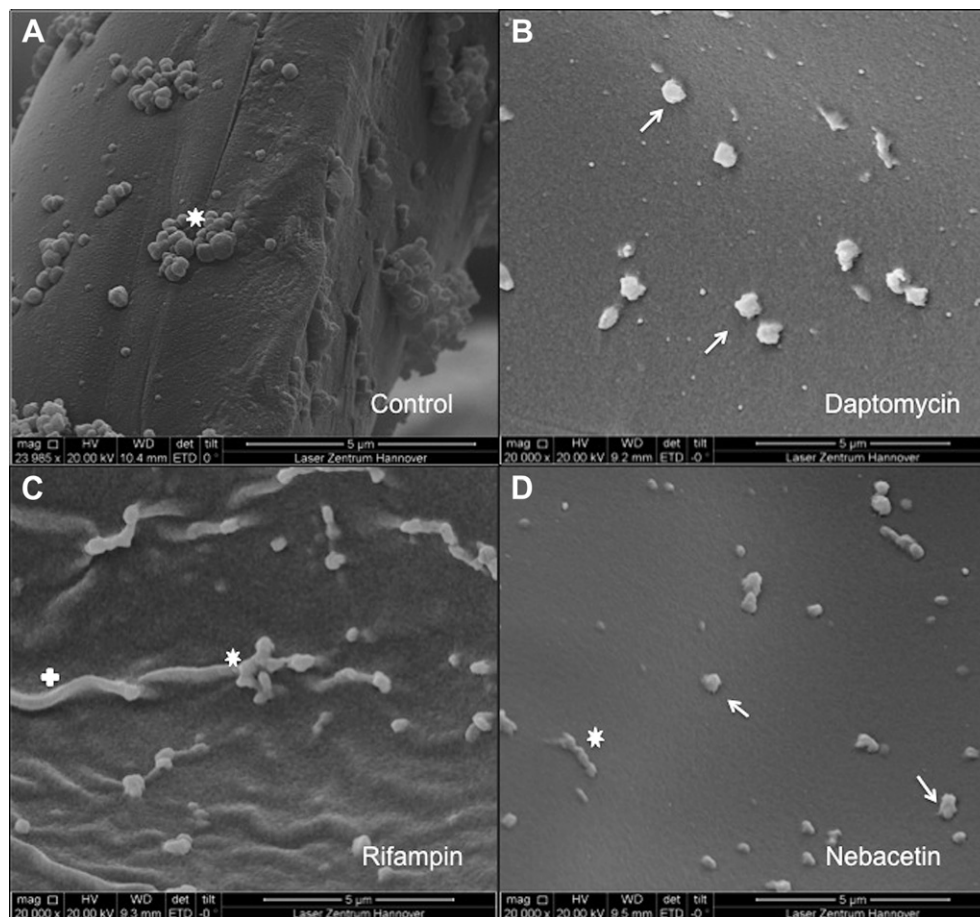
## Discussion

Nebacetin consists of two different compounds, neomycin and bacitracin. Neomycin as aminoglycoside acts on susceptible bacteria (mostly Gram-negative) presumably by irreversibly binding to the 30S ribosomal subunit, thereby inhibiting bacterial protein synthesis.<sup>15</sup> Bacitracin is a polypeptide produced by strains of *Bacillus licheniformis* and acts against cell-wall synthesis through inhibition of de-phosphorylation of C<sub>55</sub>-isoprenyl pyrophosphate, which is essential for regeneration of the lipid carrier required for the cyclic synthesis of peptidoglycan. Hence, bacitracin distorts the membrane structure due to either the removal of the lipids or direct penetration.<sup>16</sup>

Our study is the first one in English literature testing nebacetin as impregnation agent for prevention of VGI. Since 1953 both antibiotics have been widely used for skin and eye infections.<sup>17</sup>

Neomycin sulphate is indicated for the treatment of superficial infections, prophylaxis against infection in minor and postoperative wounds, adjunctive treatment of burns and management of superinfection in chronic dermatoses, stasis dermatitis or chronic leg ulcers.<sup>18</sup> However, allergic contact dermatitis (1–6% in intact skin), delayed hypersensitivity or immunoglobulin E (IgE)-mediated reactions and plasmid-mediated resistance have been reported.<sup>19</sup> Bacitracin is used for local infections, secondary pyoderma and as adjunct measure in burn treatment and in operative wounds.<sup>18</sup> Few cases of delayed hypersensitivity, IgE-mediated allergic reactions or dermatitis have been reported.<sup>19</sup> Along with neomycin (nebacetin) led to increased risk of sensitisation in the treatment of chronic ulcers.<sup>18</sup> We tested additionally the toxic effects of nebacetin as well as of the other antibiotic agents against ECs. ECs are particularly attractive for toxicity tests representing a sensitive system that maintains several phenotypic and genotypic characteristics of human cells *in vivo*.<sup>20</sup> Besides, ECs provide rapid coverage of porous synthetic graft surfaces in contact with arterial circulation (graft anastomosis).<sup>21</sup>

The results of our study support the hypothesis that nebacetin is very effective to eliminate *in vitro* VGI caused by *S. epidermidis*, *S. aureus*, *P. aeruginosa* or *E. coli*. Regarding Gram-positive bacteria, where several reports advocate the advantageous use of daptomycin, nebacetin also showed comparable efficacy. Rifampin was not as efficient as expected and additionally was high toxic against ECs. Furthermore, evaluation of the infected grafts under SEM confirmed the excellent antibacterial effect of daptomycin (Figs. 4



**Figure 5.** Example of a 24h-old biofilm of *S. aureus* in control group (A), after impregnation with daptomycin (B), rifampin (C) and nebacetin (D). The asterisks show the formed biofilm with intact organisms and the arrows represent the eliminated bacteria (lysis). Besides, in the biofilm of the rifampin-impregnated grafts the characteristic extracellular polymeric substance is presented (cross).

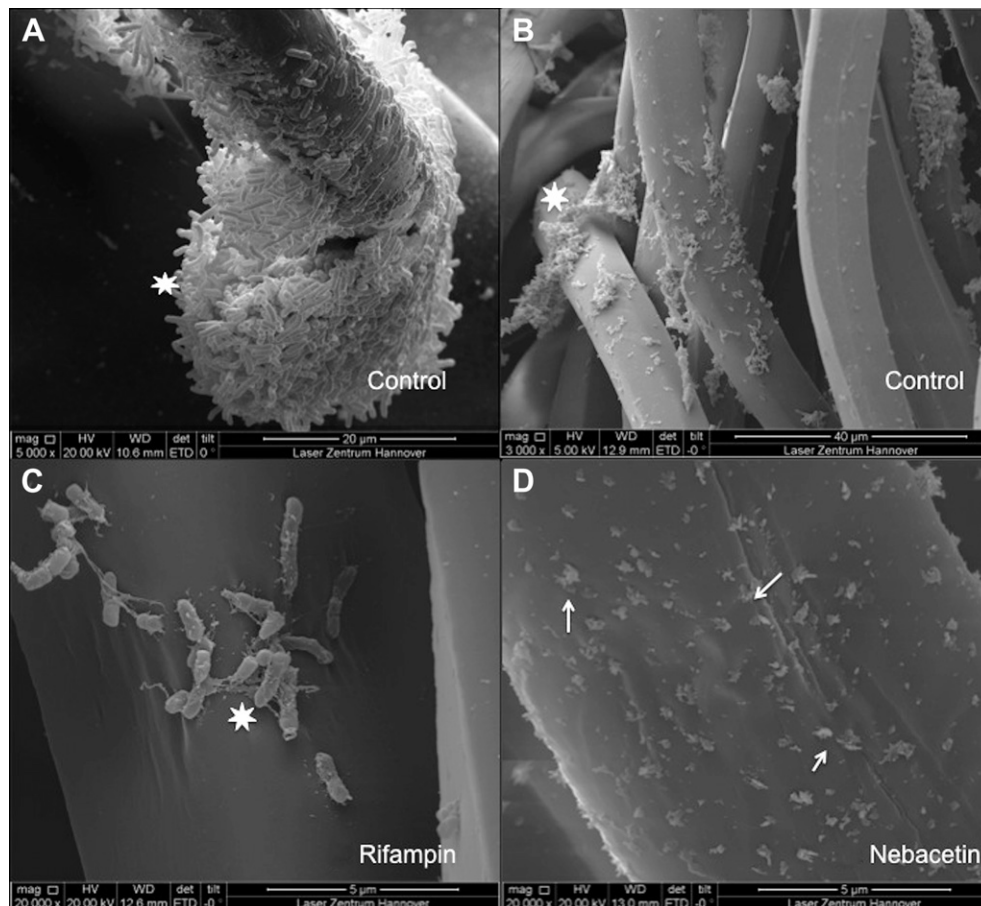
and 5) and of nebacetin even against difficult biofilms (*P. aeruginosa*) (Fig. 6). Biofilm remains the most relevant feature for the pathogenicity of bacteria colonising surgical implants, protecting them from recognition and elimination through host defence mechanisms.<sup>6</sup>

The most common agent causing VGI remains *S. epidermidis* with the characteristic exopolysaccharide biofilm.<sup>3,4,8</sup> According to Edmiston et al., prognostic factors affecting the outcomes of staphylococcal device infections are (1) the composition and structural characteristics of the biomedical device surface, (2) the selective activity of the therapeutic agent and (3) the presence or absence of exopolysaccharide biofilm.<sup>22</sup> Hence, several preventive measures have been developed to optimise penetration of biofilm and to maintain adequate levels of antibacterial substance into tissue during the surgical procedure. Common practice is the perioperative use of antibiotics and the aseptic conditions of surgery.<sup>6–8</sup> However, it remains questionable if both techniques are able to prevent bacterial adhesion and to penetrate a biofilm over a non-vascularised graft. Therefore, graft impregnation for local antibiotic release in the peri-graft tissues has been supported as an advantageous adjuvant technique, especially in patients having several risk factors for VGI (autoimmune disease, immunosuppression, diabetes mellitus, corticosteroid administration, liver disease/cirrhosis, malnutrition, etc.).<sup>23</sup> In this context, encouraging results after the use of antibiotic-impregnated polymethylmethacrylate beads for the treatment of experimental prosthetic graft infections support this concept.<sup>24</sup> However, considerations for rapid development of antibiotic resistance have been uttered.<sup>25</sup> To field this issue, Friberg et al.

demonstrated in 1359 patients, undergoing local implantation of collagen-gentamycin for the prevention of sternal wound infections, no long-term increase in the absolute incidence of aminoglycoside resistance agents.<sup>25</sup>

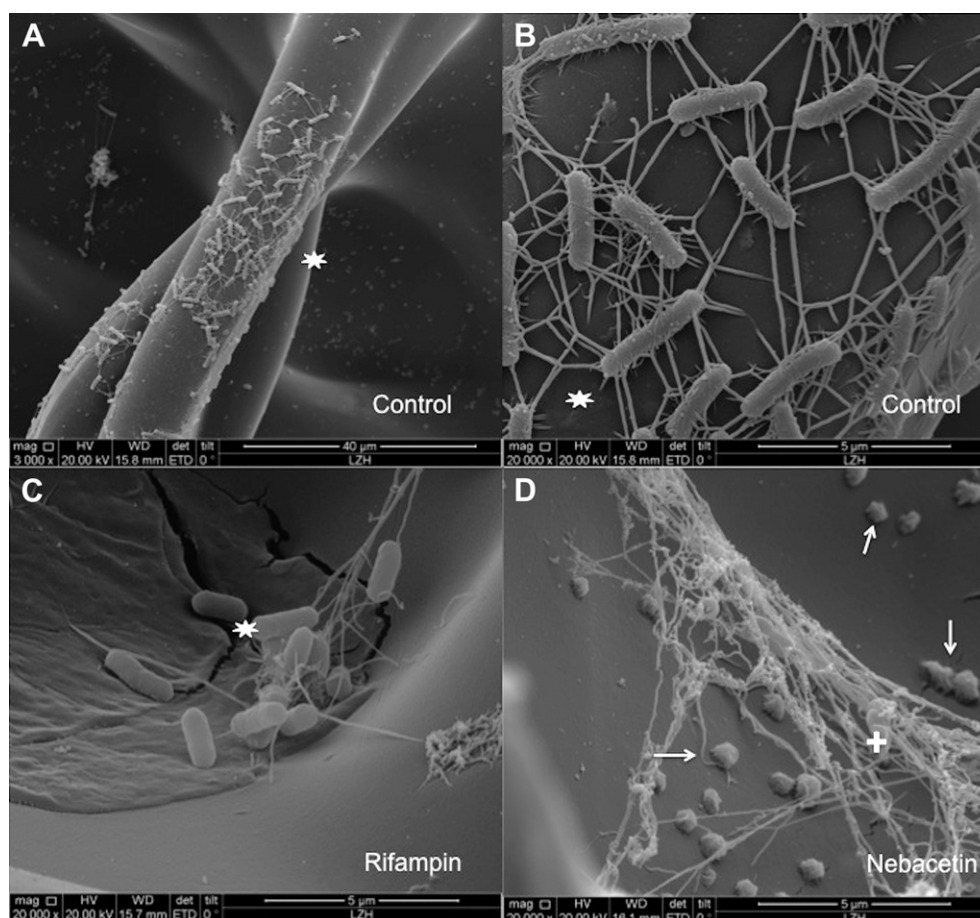
Notwithstanding on the method of graft impregnation or coating, the most relevant factor remains the effectiveness of the antibiotic agent, which should cover, ideally, both Gram-positive and -negative bacteria. Further relevant parameters are substance toxicity (systemic and local) and costs of therapy. Rifampin has a chemical property that easily links to gelatin in a form of ionic bonding and targets Gram-positive and -negative micro-organisms.<sup>26</sup> Regarding the adherence of daptomycin or nebacetin on the gelatin layer or other matrices of impregnation (e.g., cyclodextrin polymers) no data are available in English or German literature. However, antibacterial activity of rifampin has been challenged due to its effect reduction after brief exposure, especially by staphylococcal infections.<sup>8</sup> In contrast, daptomycin is a high-promising new agent due to its ability to inhibit organisms embedded in biofilms and to its great antimicrobial activity against resistant micro-organisms.<sup>8</sup> It was approved in 2003 for the non-topical treatment of skin structure infections caused by Gram-positive pathogens, including methicillin-resistant *S. aureus* (MRSA) and in 2006 for the treatment of bacteraemia.<sup>27</sup> Disadvantage remains its limited activity against exclusively Gram-positive bacteria. In this context, nebacetin is superior to rifampin and daptomycin.

Unclear, however, is whether nebacetin still acts more effectively compared to rifampin or daptomycin combination therapies. Where Cirioni et al.<sup>8</sup> addressed that combination of rifampin and



**Figure 6.** Example of a 24h-old biofilm of *P. aeruginosa* in control group (A,B), after impregnation with rifampin (C) and nebacetin (D). The asterisks show the formed biofilm with intact organisms and the arrows represent the eliminated bacteria (lysis).





**Figure 7.** Example of a 24-h-old biofilm of *E. coli* in control group (A,B), after impregnation with rifampin (C) and nebacetin (D). The asterisks show the formed biofilm with intact organisms and the arrows represent the eliminated bacteria (lysis). Of note in nebacetin-impregnated graft, albeit the characteristic network of the micro-organism (cross) has been built, all bacteria have been eliminated.

daptomycin showed efficacies significantly higher than the respective of each single compound, other groups showed indifference or antagonism when used against MRSA.<sup>28</sup> More promising results have been published after a combination of rifampin with linezolid for the treatment of infections caused by *S. aureus* or MRSA strains. In these series, linezolid–rifampin treatment prevented the emergence of rifampin-resistant mutants.<sup>26,28</sup> However, an overview of the *in vitro* data for rifampin combination therapies against staphylococci demonstrated rather antagonism or indifference than synergy.<sup>28</sup>

Another interesting outcome in this study was the considerably high toxic effects of rifampin against ECs. Rifampin's toxicity against ECs may explain the observed necrosis at the anastomoses in 25% of gelatin-sealed grafts soaked with rifampin as reported by Schneider et al. in a dog model with MRSA and *E. coli* infection, albeit this remains a speculation.<sup>29</sup> In that model, histological evaluation of the anastomoses revealed that the necrosis was not due to bacterial colonisation.<sup>29</sup> In any case, the toxic effect of antibiotics on ECs might be crucial for the long-term patency, especially in small-calibre prostheses and this requires further investigation.

We do recognise a number of limitations in our study. We conducted this *in vitro* study as a first step in order to test an unknown in vascular surgery antibiotic agent (nebacetin). However, *in vivo* analysis is still missing and this underpowers the outcome of this model. We did not assess emergence of antibiotic resistance of the micro-organisms and the observation time (24 h) was short to assess the efficacy of antibiotics against later post-operative graft colonisation. Although nebacetin and daptomycin

showed comparable efficacy against staphylococcal infections, it remains unclear if nebacetin is also effective against MRSA. Finally, biofilms were assessed in a static and not dynamic experimental model and the possible impact of blood or peritoneal fluid flow on biofilm formation could not be assessed.

In conclusion, the results of this study support the superiority of nebacetin for the early prevention of *in vitro* VGI caused by either gram-positive or -negative micro-organisms: (1) the more effective bacterial eradication in comparison to rifampin, (2) the comparable to daptomycin antibacterial effect against non-resistant staphylococcal infections, (3) the excellent antibacterial activity against *P. aeruginosa* and *E. coli*, (4) the absence of any toxicity against ECs and, lastly, (5) the combination of two compounds in one antibiotic agent (lower costs) remain its advantageous characteristics.

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#### Conflict of Interest

This study was supported by internal funds. BBraun provided the grafts for this study and has paid Dr Bisdas a consulting fee for a lecture.

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